

Generating Tools to Study Protein Quality Control Using *Saccharomyces cerevisiae*

An Honors Thesis (HONR 499)

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Abstract

Proteins can occasionally behave aberrantly within the cell and cause a variety of physiological issues. Multiple pathways exist that resolve this problem and allow the cell to continue normal protein production. Endoplasmic Reticulum (ER)-Associated Degradation (ERAD) and Inner Nuclear Membrane-Associated Degradation (INMAD) are pathways that remove these misfolded and otherwise aberrant proteins. Dysfunction in either system may result in numerous pathologies, including those relating to cholesterol homeostasis and neurodegeneration. When misfolded proteins accumulate in the endoplasmic reticulum (ER stress), some of these misfolded proteins are unable to be removed. We hypothesize that there are proteins that work in both pathways (ER stress recovery and ERAD/INMAD), and when present in excess, can restore degradation. Using the model organism *Saccharomyces cerevisiae*, proteins involved these pathways will be studied. First, a plasmid harboring the gene for a known ERAD substrate that engages the translocon was created. Second, four unique yeast strains were created, each harboring a combination of gene knockouts that encode enzymes involved in both ERAD and INMAD degradation pathways. Understanding these protein quality mechanisms may facilitate the development of improved therapeutics for human disease.

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Process Analysis

This project has revealed more about myself than I had expected. When I first began working in Dr. Rubenstein's lab two years ago, it was quite an unfamiliar environment. The only lab experience I had was from the lab sections of my major classes, which offered a regimented and formulaic dynamic. Protocol within the weekly experiments was clearly defined by the instructor, and my actions were always closely watched to ensure I didn't make any errors. Extracurricular research was a stark contrast to this system. Each week, my PI outlined what needed to be done in each step, and then I was on my own to complete each objective. Even though I may have failed to get adequate results a few times, this process eventually revealed to me the resiliency that is needed to be successful in the field of cellular and molecular biology. Failure allowed me to see what I did wrong, and create a plan to ensure it didn't happen again. It wasn't failure in the sense that we as students often believe, but an opportunity to learn. It not only cultivated resiliency, but determination and critical thinking as well.

This project is full of blood, sweat and tears, and I am couldn't be more proud of the final outcome. I have learned so much about the field of cellular and molecular biology, as well as how to write professionally. The character qualities that have been reinforced throughout this experience will follow me as I enter medical school in the Fall, and beyond. I will have to apply that same determination as before, and refuse to accept perceived failure.

Introduction

Protein quality control within the eukaryotic cell is dependent upon the ubiquitin proteasome system, or UPS [1]. A number of different enzymes work in conjunction to target and destroy damaged, misfolded, or aberrant proteins. Before degradation can occur, ATP hydrolysis must fuel the covalent linkage of ubiquitin molecules to E1 ubiquitin-activating enzymes. The ubiquitin molecules can be transferred to one or more E2 ubiquitin-conjugating enzymes, which then bind to E3 ubiquitin ligases. The E3s then bind to substrates (misfolded, damaged, or otherwise aberrant proteins), and mediate the transfer of ubiquitin from E2s onto targeted proteins. After polyubiquitylation, these aberrant proteins can then be degraded by a protease located in the cytosol, known as the proteasome [1-3].

Endoplasmic Reticulum (ER)-Associated Degradation (ERAD) is the targeting of ER-resident misfolded or otherwise aberrant proteins for degradation [1, 4, 5]. Depending on where the degradation signal (the degron) lies within the aberrant protein, ERAD-C, ERAD-M, ERAD-L, ERAD-T or Inner Nuclear Membrane-Associated Degradation (INMAD) could be invoked to remove it (Figure 1). Degrons that lie within the cytosolic portion of a protein undergo ERAD-C (cytosol), which is mediated by the E3 ubiquitin ligase Doa10. These proteins can be either transmembrane with cytosolic portions or soluble cytosolic proteins that come into contact with the ER. If the degradation signal lies within a transmembrane segment of a protein, ERAD-M (membrane) will be initiated along with the E3 Hrd1 to remove the protein. Proteins with degrons within the lumen are also Hrd1 substrates and utilize the ERAD-L (lumen) pathway for removal [4].

As proteins enter the endoplasmic reticulum for processing or secretion from the cell, they must first pass through a protein channel known as the translocon [6]. Proteins that become

stuck within this channel must be removed in order to maintain proper homeostasis. Rubenstein et al. discovered that the ubiquitin ligase that targets these substrates for ubiquitylation and subsequent degradation via the proteasome is Hrd1 via the ERAD-T (translocon) pathway [7]. When a protein blocks this channel, translocation of other proteins into the ER may become hindered. It is not until Hrd1 catalyzes the polyubiquitylation of the aberrant protein and the protein is subsequently degraded by the proteasome that functional translocation can be reestablished. There are numerous associated proteins that work in the ERAD-C, ERAD-L, and ERAD-M pathways alongside Doa10 and Hrd1; however, the proteins that function with Hrd1 in the ERAD-T pathway remain under study [6, 7, 8]. At least one medically relevant protein, apolipoprotein B (apoB) persistently engages the translocon in humans. ApoB is a component of low-density lipoproteins (LDLs, or “bad cholesterol”) within the human circulatory system. ApoB partially traverses the translocon and awaits lipid binding to its ER lumenally exposed portion. Following lipid binding, apoB passes completely through the translocon and assembles with the bound lipids into an LDL particle. If lipid binding is prevented, Hrd1 recognizes and ubiquitylates apoB, targeting it for degradation. Further understanding of this pathway could lead to improved treatment of hypercholesterolemia.

Aberrant proteins within the inner nuclear membrane are degraded by a pathway known as INMAD [9]. A complex of integral membrane proteins catalyze the ubiquitylation of these proteins, which allows for their degradation by the proteasome. Three subunits (Asi1, Asi2, and Asi3) form a holoenzyme called the Asi complex that acts as the E3 ubiquitin ligase in this pathway. This pathway, like the other ERAD pathways, promotes homeostasis within the cell by promoting the removal of damaged or misfolded proteins [9, 10]. Little is known about whether or how the ERAD and INMAD E3 ubiquitin ligases work in conjunction with one another.

Systematic combinations of E3 gene knockouts (created in this study) will provide a way to study the function and interactions of the Asi complex within the cell.

Protein quality control mediated by the UPS is critical for cell survival. ERAD or UPS dysfunction is a common etiology of many human diseases [16, 17]. Alzheimer's disease and Parkinson's disease have been proposed to be the result of malfunction of the ubiquitin proteasome system at the ER membrane and the subsequent accumulation of aberrant proteins. This system can also be a future therapeutic target for conditions like cystic fibrosis. A single amino acid mutation causes the creation of a near-functional protein; however, the ERAD pathway catalyzes its degradation. Further, ERAD-T of apoB may be a therapeutic target for hypercholesterolemia. Discovery of the proteins that function in these pathways may yield novel therapeutic targets for these conditions.

Protein quality control pathways can be studied *in vivo* using *Saccharomyces cerevisiae*, or baker's yeast. *S. cerevisiae* is an optimal organism for this study due to its extensively mapped genome, short doubling time of approximately 90 minutes, and conservation of the UPS and ERAD systems with other eukaryotic organisms [1, 4, 14, 15]. Thus, this organism provides an excellent model for basic biology relevant to human health and disease.

The first identified degron that targets a protein for UPS-dependent degradation is *Deg1*, the N-terminal 67 amino acids of the transcriptional repressor MAT α 2. MAT α 2 is a substrate of the Doa10-mediated ERAD-C system [4, 11, 12]. When *Deg1* is artificially linked to an otherwise stable soluble cytosolic protein (e.g. green fluorescent protein) or transmembrane protein (e.g. Vma12), it causes the fusion protein to be removed via ERAD-C [13]. Sec62, a transmembrane protein with similar membrane topology to Vma12, was also expected to be converted to a Doa10 substrate when *Deg1* was fused to the cytosolic N-terminal portion of the

protein. However, Rubenstein et al. discovered that the *Deg1*-Sec62 protein became a Hrd1 substrate, following aberrant interaction with the translocon [7].

The fusion of *Deg1* to the transmembrane protein Sec62 causes a portion of the protein to move into – and likely stall within – the translocon [7; Figure 2]. The Sec62 protein is comprised of two transmembrane segments that are likely integrated into the ER membrane via co-translational translocation. The *Deg1* degron is located on the cytosolic N-terminus of the protein and triggers aberrant engagement with the translocon. Hrd1 is then recruited based on this interaction and catalyzes the initiation of ERAD-T [7]. This mechanism is known to be different than the Doa10-dependent degradation of *Deg1*-containing proteins, because a mutation in *Deg1* that prevents Doa10-dependent degradation of ERAD-C substrates does not similarly prevent Hrd1 degradation of the *Deg1*-Sec62 protein [7].

A unique plasmid housing the *Deg1*-Sec62 coding sequence in frame with the Ura3 enzyme (which catalyzes an essential step in the uracil biosynthesis pathway) was created to further study the ERAD-T pathway (Sheldon Watts and Eric Rubenstein, unpublished data). The gene encoding *Deg1*-Sec62-Ura3 is designed to be amplified by PCR and integrated into the yeast genome. To select for yeast cells that possess *Deg1*-Sec62-Ura3, the *natMX4* gene (which confers resistance to the antibiotic nourseothricin) was linked to this gene (as a completely separate gene) in this study; growth of cells in the presence of nourseothricin would confirm presence of the DNA encoding *Deg1*-Sec62-Ura3. The *Deg1*-Sec62-Ura3 gene product will then allow for selection on the basis of yeast growth in the absence of uracil and help pinpoint genes that work in the ERAD-T pathway. Ura3 catalyzes a reaction in the uracil biosynthesis pathway. Yeast cells with mutations that impair ERAD-T (i.e. fail to degrade *Deg1*-Sec62-Ura3) will selectively grow on media that lacks uracil (Figure 3). The Ura3 protein can also be exploited in

a counter-selection growth assay involving the compound 5-Fluoroorotic Acid (5-FOA). In the presence of a functional Ura3 protein, 5-FOA is converted to the toxic compound 5-fluorouracil (5-FU). Therefore, if ERAD-T is not functioning properly and *Deg1*-Sec62-Ura3 protein is stabilized, 5-FU will be created, and the cells will not survive.

The counter-selection growth assay can be used to identify genes that influence ERAD-T. ERAD-T is impaired under conditions of ER stress, which is characterized by elevated levels of misfolded proteins in the ER lumen (Figure 3; Bryce Buchanan, Eric Rubenstein, and Mark Hochstrasser, unpublished data). It is hypothesized that a protein that functions in ERAD-T may also function in the ER stress response. Overexpression of this protein may rescue the ERAD-T pathway under ER stress conditions. Using the previously described counter-selection strategy, stabilization of *Deg1*-Sec62-Ura3 within the translocon and subsequent creation of 5-FU during conditions of ER stress will impair cell survival. A gene that functions in both ERAD-T and ER stress response may be identified on the basis of restored cell growth (i.e. restored ERAD-T) under ER stress conditions if the gene is overexpressed.

The importance of protein quality control cannot be understated. Malfunctions in ERAD have the potential to cause a variety of harmful etiologies. The plasmid and yeast strains created for these studies can be used to investigate these mechanisms. With a better understanding of these pathways lies the potential for the development of new therapeutic strategies.

Goals

1. Generate a plasmid encoding a model ERAD-T substrate (*Deg1*-Sec62-Ura3) and a gene conferring nourseothricin resistance (natMX4).
2. Generate yeast strains expressing and lacking all combinations of ERAD and INMAD ubiquitin ligases (Doa10, Hrd1, and Asi complex).

Materials and Methods

Creation of plasmid containing Deg1-Sec62-Ura3 and natMX4

The parent plasmids pVJ32 (p414-MET25-*Deg1*-*Sec62*-*Ura3*; vector plasmid) and pVJ132 (pAG25; insert plasmid) were stored in *E. coli* cells at -80°C. These *E. coli* were first streaked for isolation on Luria Broth plates that contained 50 µg/mL ampicillin, and incubated for 24 hours at 37°C. An individual colony from each plate was inoculated into 4 ml of liquid Luria Broth with ampicillin. The plasmid DNA was then isolated from these inoculates using the Promega miniprep kit and protocol. These DNA preparations were then digested with the restriction enzyme *EagI* overnight at 37°C (5 µL miniprep DNA, 1 µL of *EagI*, 2.5 µL 10X NEBuffer, and 16.5 µL water). After digestion, the vector plasmid was incubated with Antarctic phosphatase for one hour at 37°C (3 µL of Antarctic phosphatase buffer and 1 µL Antarctic phosphatase were added directly to the vector digest). The digested samples were then separated on a 1% agarose gel at 100 volts in TAE (Tris-acetate ethylenediaminetetraacetic acid (EDTA)) buffer for approximately 45 minutes. From the gel, a 7673 bp fragment (vector) and 1314 bp fragment (insert) were isolated and purified using the Qiagen gel purification kit and protocol. The vector and insert fragments were eluted in 30 µL of elution buffer. The purified fragments were then ligated using T4 DNA ligase in 1X ligation buffer in 20 µL ligation reactions, and incubated at 23°C overnight. Four ligation reactions were created. The first reaction included 2 µL of insert DNA, 2 µL of vector DNA, and 1 µL of T4 DNA ligase. The second reaction included 15 µL of insert DNA, 2 µL of vector DNA, and 1 µL of T4 DNA ligase. The third reaction included 2 µL of vector DNA and 1 µL of T4 DNA ligase (and no insert DNA). The fourth reaction included 2 µL of vector DNA (and no insert DNA or T4 DNA ligase). The ligation reaction products were then transformed into chemically competent *E. coli* cells by

mixing 10 μ L of the ligation reaction products and 25 μ L of cells. These components were then placed on ice for approximately 1 hour and 45 minutes, incubated in a 42°C water bath for 30 seconds, and returned to the ice for two minutes. Next, 1 mL of warm (37°C) Luria Broth was added to each tube, which was incubated at 37°C for 30 minutes. The cells were pelleted via centrifugation (13,000 x g for 5 minutes) and resuspended in approximately 100 μ L of Luria Broth. These cells were then spread on Luria Broth plates with ampicillin and incubated overnight at 37°C.

The recombinant plasmid DNA of candidate colonies was isolated via the Promega miniprep kit and protocol and digested with the restriction enzyme EagI in NEBuffer 3.1, as described above. Digested DNA was analyzed on a 1% agarose gel to screen for the presence of both the vector (7673 bp) and insert (1314 bp) fragments. Candidate samples that contained both fragments were then screened for orientation of the insert using a high-fidelity version of the restriction enzyme NcoI (5 μ L miniprep DNA, 1 μ L of NcoI-HF, 2.5 μ L of 10X CutSmart Buffer, and 16.5 μ L of water). The samples were incubated overnight at 37°C and analyzed on another 1% agarose gel. If the insert fragment was ligated such that the natMX4 gene and the *Deg1*-Sec62-Ura3 gene were oriented in the same direction (i.e. transcribed from the same strand), the expected NcoI-generated fragments would be 6212, 1461, and 1324 bp. By contrast, if the insert fragment was ligated in the opposite direction of the *Deg1*-Sec62-Ura3 gene, the expected NcoI-generated fragments would be 5790, 1736, 1461 bp. Candidates that displayed appropriate banding patterns were stored as glycerol permanent stocks at -80°C by combining 1 mL of culture with 500 μ L of 75% glycerol.

Generation of yeast strains with knockout of ASI3 gene

In this procedure, a fragment of DNA containing the natMX4 gene was PCR-amplified

from pAG25 (pVJ132) and integrated into the genome of yeast strains VJY22 (*hrd1Δ*), VJY102 (*doa10Δ*), VJY305 (*hrd1Δ doa10Δ*), and VJY97 (WT). Yeast strains used in this study are presented in Table 1. This fragment containing natMX4 was amplified with sequences homologous to sequence flanking the *ASI3* gene and integrated into the yeast genome via homologous recombination.

Amplification of the natMX4 gene was achieved via PCR from the pVJ132 plasmid. The primers VJR274 and VJR275 (See Table 2 for primer sequences) were used to amplify the natMX4 gene from the plasmid. The 5' ends of these primers have sequences of nucleotides that are homologous (identical) to nucleotides in the yeast genome immediately upstream and downstream of the *ASI3* gene coding sequence. These sequences will promote homologous recombination at the *ASI3* locus, replacing that gene with the natMX4 gene. To generate the PCR fragment, the following ingredients were combined and separated into 50 μ L aliquots: 20 μ L of 10X ThermoPol buffer, 4 μ L of 10 mM dNTPs, 4 μ L of pVJ132 miniprep, 1 μ L of *Taq* polymerase, 4 μ L of 50 mM primer VJR 274, 4 μ L of 50 mM primer VJR 275, and 163 μ L of molecular biology grade water. PCR was performed using the following conditions: segment 1 (1 cycle: 95°C for 5 minutes), segment 2 (35 cycles: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute 30 seconds), segment 3 (1 cycle: 72°C for 10 minutes), and a final hold at 10°C. 1 μ L of the PCR product was separated on a 1% agarose gel, and the expected 1464 bp band was observed. The PCR fragment was column-purified using the Qiagen Gel Purification kit and the column purification protocol.

To promote uptake of the PCR product into yeast cells and subsequent homologous recombination of the natMX4 gene at the *ASI3* locus, a high-efficiency yeast transformation protocol was performed. The yeast strains VJY22, VJY102, VJY305, and VJY97 were first

grown on yeast extract peptone dextrose (YPD) agar plates, incubated overnight at 30°C, and then inoculated into 5 mL of YPD broth for overnight incubation at 30°C. The optical density at 600 nm (OD_{600}) of the overnight cultures was determined, and the cultures were subsequently diluted to an OD_{600} of 0.2 in 50 mL of YPD liquid. The samples were then placed in a shaker at 30°C and allowed to reach mid-logarithmic growth phase (an OD_{600} of approximately 1.0). Once this density was reached, the cells were centrifuged at 1,500 x g for seven minutes in a 50 mL conical tube. The supernatant was decanted, and the pellet was then resuspended in 25 mL of sterile water and centrifuged again under the same conditions. The supernatant was decanted. The cells were resuspended in 1 mL of 100 mM Lithium Acetate and transferred to a 1.5 mL microcentrifuge tube. This cell suspension was then centrifuged at 6,800 x g for two minutes, and the supernatant was removed by pipetting. 400 μ L of 100 mM lithium acetate was added to the tube, and the cell pellet was again resuspended. The cells were suspended by vortexing, and 100 μ L of the suspension was aliquoted to a new microcentrifuge tube for each transformation. The suspension was centrifuged at 6,800 x g for two minutes, and the supernatant was removed by pipetting. 240 μ L of 50% (w/v) polyethylene glycol, 36 μ L of 1.0 M lithium acetate, and 20 μ L of freshly denatured (5 mg/mL) salmon sperm DNA were added to each transformation tube. Varying amounts of PCR product (0 μ L, 4 μ L, or 40 μ L) were added to individual transformation tubes. The samples were then mixed via pipetting and incubated in a 42°C water bath for 40 minutes. The samples were centrifuged at 10,000 x g for 30 seconds, and the supernatant was removed via pipetting. 50 μ L of sterile water was added to each tube, and the pellet was resuspended. Resuspended cells were plated onto YPD growth media. After one day of growth, the colonies were replica-plated onto YPD plates with nourseothricin (100 μ g/mL) using sterile velvet sheets.

After four days of growth on the selective media, up to 16 candidates from each transformation were isolated for single colonies on YPD plates with nourseothricin and allowed to incubate overnight at 30°C. These candidates were then inoculated into 4 mL of YPD and were allowed to incubate overnight, rotating at 30°C. An untransformed parental control strain was also streaked and inoculated in parallel with the candidates.

To screen for integration of natMX4 at the *ASI3* locus, DNA was isolated from respective colonies of the transformed and parental control strains. 1 mL of saturated culture was transferred to a 2 mL screw-cap microcentrifuge tube and centrifuged at 2,700 x g for 2 minutes. The supernatant was removed, and 500 µL of sterile water was added to each cell pellet. The pellet was resuspended and centrifuged using the same conditions. The supernatant was removed, and the cell pellets were resuspended in 200 µL of Buffer A (2% Triton X-100, 1% sodium dodecyl sulfate, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA). Approximately 200 µL of acid washed glass beads were added to each tube, followed by the addition of 200 µL of phenol:chloroform:isoamyl alcohol (25:24:1) under a fume hood. The tubes were sealed with parafilm and agitated in an MP Biomedicals FastPrep-24 for 60 seconds at 5 meters/second at 4°C. Under the hood, the tubes were opened, 200 µL of Buffer A was added, and the tubes were re-sealed with parafilm. After manually vortexing each tube for 10 seconds, the tubes were centrifuged for 5 minutes at 21,000 x g. Under a hood, 200 µL of the aqueous layer (which contained the DNA) was transferred to a fresh 1.5 ml microcentrifuge tube. 1 mL of 95% ethanol was then added to each tube, and the DNA was allowed to precipitate overnight at -20°C. The samples were centrifuged for 10 minutes at 21,000 x g at 4°C, and the supernatant was removed via pipetting. The residual ethanol was allowed to evaporate out under a fume hood for 5

minutes. The pellet was resuspended in 25 μ L of Tris-EDTA buffer (TE buffer; 10 mM Tris base, 0.5 mM EDTA, pH 8.0).

Using this yeast genomic DNA, two sets of PCR reactions were performed to confirm integration of natMX4 at the *ASI3* locus, one at the 5' end of the *ASI3* locus and one at the 3' end. For the 5' PCR, each reaction mixture included 2.5 μ L of 10X ThermoPol buffer, 0.5 μ L of 10 mM dNTPs, 0.5 μ L of primer VJR276, 10 μ L of primer VJR277, 0.5 μ L of primer VJR246, 0.125 μ L of *Taq* polymerase, and 19.875 μ L of molecular biology grade water. To each tube, 0.05 μ L of the yeast genomic DNA was added, and PCR was performed using the following cycles: segment 1 (1 cycle: 95°C for 5 minutes), segment 2 (35 cycles: 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute), segment 3 (1 cycle: 72°C for 10 minutes), and a final hold at 10°C. The PCR product was then separated on a 1% agarose gel. The expected product sizes were 820 bp (for cells harboring *asi3 Δ ::natMX4* mutation) or 649 bp (for cells harboring wild type *ASI3*). For the 3' PCR, each reaction tube included 2.5 μ L of 10X ThermoPol buffer, 0.5 μ L of 10 mM dNTPs, 0.5 μ L of primer VJR278, 0.5 μ L of primer VJR279, 0.5 μ L of primer VJR249, 0.125 μ L of *Taq* polymerase, and 18.875 μ L of molecular biology grade water. To each tube, 0.05 μ L of the yeast genomic DNA was added, and PCR was performed with the same cycles used for the 5' PCR reaction, described above. The PCR products were then separated on a 1% agarose gel. The expected product sizes were 808 bp (for cells harboring the *asi3 Δ ::natMX4* mutation) or 623 bp (for cells harboring wild type *ASI3*). Once the candidates had been confirmed using both 5' and 3' three-primer PCR, successful clones were stored at -80°C as glycerol permanent stocks by combining 500 μ L of 75% glycerol and 1 mL of culture.

Results

Generation of plasmid encoding Deg1-Sec62-Ura3:natMX4

The protein *Deg1-Sec62-Ura3* (DSU) is a useful tool for the study of the ERAD-T pathway because the Ura3 component, linked to the C-terminus of the protein, will allow for selection of cells that exhibit differing abilities to perform ERAD-T. Cells that fail to degrade DSU will grow selectively in the absence of uracil, while cells that can degrade DSU will grow selectively in the presence of 5-FOA (Figure 3). This plasmid will be used in future experiments involving collections of yeast strains systematically lacking or overexpressing particular genes to identify those that work within the ERAD-T pathway.

The creation of a plasmid containing *Deg1-Sec62-Ura3* and the *natMX4* gene (which confers nourseothricin resistance) occurred through restriction enzyme-mediated subcloning, whereby a fragment containing the *natMX4* gene was inserted downstream of the DSU gene. Using the restriction enzyme *EagI*, the 1314 bp *natMX4* fragment was isolated from a parental plasmid (pAG25/ pVJ132; Figure 4), and inserted into the *EagI*-linearized 7673 bp DSU parental vector (p414-MET25-*Deg1-Sec62-Ura3*/ pVJ132; Figure 5) that also contained the ampicillin resistance gene, *ampR*. The desired recombinant plasmid is illustrated in Figure 6. Digested plasmid fragments prepared to generate the recombinant DNA molecule were separated by agarose gel electrophoresis; the insert and vector fragments were gel-purified (Figure 7). Plasmids from eight candidate colonies were isolated and digested with the restriction enzyme *EagI* to determine proper introduction of the *natMX4* gene. Following *EagI* digest, two of the plasmids exhibited DNA molecules of sizes consistent with the presence of both insert and vector fragments (7673 bp and 1314 bp; *EagI* digest of parental plasmids and one successful clone is illustrated in Figure 8, lanes 1-3). Because only one enzyme was used to generate the

insert and vector fragments, the insert fragment could have been introduced in one of two orientations. Therefore, these plasmids were subsequently digested with NcoI to assess the orientation of the insert fragment (Figure 8, lanes 4-6). In both candidates, the insert displayed reverse orientation (that is, the natMX4 gene was transcribed from the opposite strand as DSU); the NcoI-generated products were 5790, 1736, and 1461 bp. No candidates exhibited the insert in the same orientation as the DSU gene. Because the natMX4 gene was not designed to be in frame with the DSU gene, either orientation is acceptable for downstream applications. One candidate (Candidate #3) was preserved as the final recombinant plasmid. This plasmid (p414-MET25-Deg1-Sec62-Ura3_natMX4_revori) was given the name pVJ519.

Generation of asi3Δ::natMX4 strains

The replacement of *ASI3* (a gene encoding a subunit of the Asi ubiquitin ligase complex) with the natMX4 gene was achieved through PCR amplification of natMX4 from a plasmid (pAG25/ pVJ132; Figures 4 and 9); transformation of this PCR product into yeast strains VJY22 (*hrd1Δ*), VJY102 (*doa10Δ*), VJY305 (*hrd1Δ doa10Δ*), and VJY97 (WT); and subsequent integration via homologous recombination. The PCR product included DNA homologous to sequences upstream and downstream of the *ASI3* gene; this encouraged recombination of natMX4 at the *ASI3* locus. After transformation, the colonies were grown on media that contained nourseothricin to select for cells that possess the natMX4 gene. Correct integration of the natMX4 gene was assessed using two sets of three-primer PCR reactions. One reaction was performed at the 5' end of the integration site, and the other at the 3' end (Figures 10-12). In both PCR reactions, one primer in the set is homologous to DNA outside of the integration site, one primer is homologous to an internal sequence within the original *ASI3* gene, and another is homologous to the new integrated sequence. When these PCR products are separated on an

agarose gel, band sizes vary depending on whether the natMX4 gene has integrated at the *ASI3* locus. Based on this analysis, the natMX4 gene was correctly inserted at the *ASI3* locus in one of sixteen *doa10Δ* (VJY22) candidates, one of sixteen *hrd1Δ* (VJY102) candidates, two of sixteen *doa10Δ hrd1Δ* (VJY305) candidates, and fifteen of sixteen WT (VJY97) candidates. Strains in which replacement of *ASI3* with natMX4 was verified were preserved for future experimentation.

Discussion

A plasmid containing the model ERAD-T substrate *Deg1*-Sec62-Ura3 and the nourseothricin resistance gene *natMX4* was created. This plasmid will be used in future experiments to determine proteins that work in both the ERAD-T and ER stress recovery pathways. Further, yeast strains lacking all combinations of ERAD and INMAD protein quality control ubiquitin ligases (Doa10, Hrd1, and Asi complex) were generated. These strains will be used to study the functions and interactions of the quality control pathways mediated by these enzymes.

When the Doa10-dependent degron, *Deg1*, was fused to the Sec62 transmembrane protein, Rubenstein et al. found that Hrd1 catalyzed its subsequent degradation [7]. The interaction between the protein *Deg1*-Sec62 and the translocon allow for Hrd1 recognition. The protein apoB acts similarly when it is translocated into the ER membrane. It stalls within the translocon preceding LDL assembly, awaiting its lipid binding partner [18, 19]. If this protein does not interact with lipids and remains lodged in this channel, Hrd1 ubiquitylates it and catalyzes its destruction via the proteasome [7]. While this mechanism is not yet well understood, the protein *Deg1*-Sec62 serves as a potential model for future study of this degradation mechanism.

The accumulation of proteins within the lumen of the endoplasmic reticulum (also known as ER stress) is a cause of numerous etiologies [20]. Under ER stress conditions, the function of the ERAD-T pathway is limited. One hypothesis is that a protein (or multiple proteins) functions in both the ER stress response and ERAD-T. During ER stress, this protein may preferentially function to reduce the burden of misfolded proteins, limiting its availability to function in ERAD-T. If expressed at high levels, it is possible that this gene product could function in both

of these pathways, even under conditions of stress. Systematic overexpression of each gene of the yeast genome may yield a protein, or proteins, that can rescue the ERAD-T mechanism during ER stress.

The *Deg1*-Sec62-Ura3 protein will allow for a growth-based investigation of ERAD-T. In the presence of the compound 5-FOA, the Ura3 enzyme produces 5-FU. Under conditions of ER stress, *Deg1*-Sec62-Ura3 is stabilized within the translocon, and yeast cells are unable to grow in the presence of 5-FOA (due to the generation of 5-FU). However, if ERAD-T is rescued by overexpression of a specific protein, cells will be able to grow because the Ura3-containing protein would be degraded through the ERAD-T pathway. Future work will be performed to identify genes that, when overexpressed, restore ERAD-T during ER stress.

The ubiquitin ligase holoenzyme, Asi, is proposed to operate in a quality control pathway parallel to ERAD at the inner nuclear membrane [10]. Yeast strains with all combinations of knockouts of genes encoding the three ubiquitin ligases of the ER and inner nuclear membrane (Doa10, Hrd1, and Asi complex) will be useful tools in investigating the mechanisms, interactions, and substrates of these pathways.

Highly conserved mechanisms of protein quality control are vital for cellular homeostasis. Here, tools were generated to study mechanisms of protein quality control in the model organism *S. cerevisiae*. Understanding these pathways could catalyze the development of therapeutic strategies for chronic medical conditions that are a result of ERAD or UPS malfunction, such as Alzheimer's disease, cystic fibrosis, cholesterol-related pathology, and Parkinson's disease [16, 17].

Figures and Tables

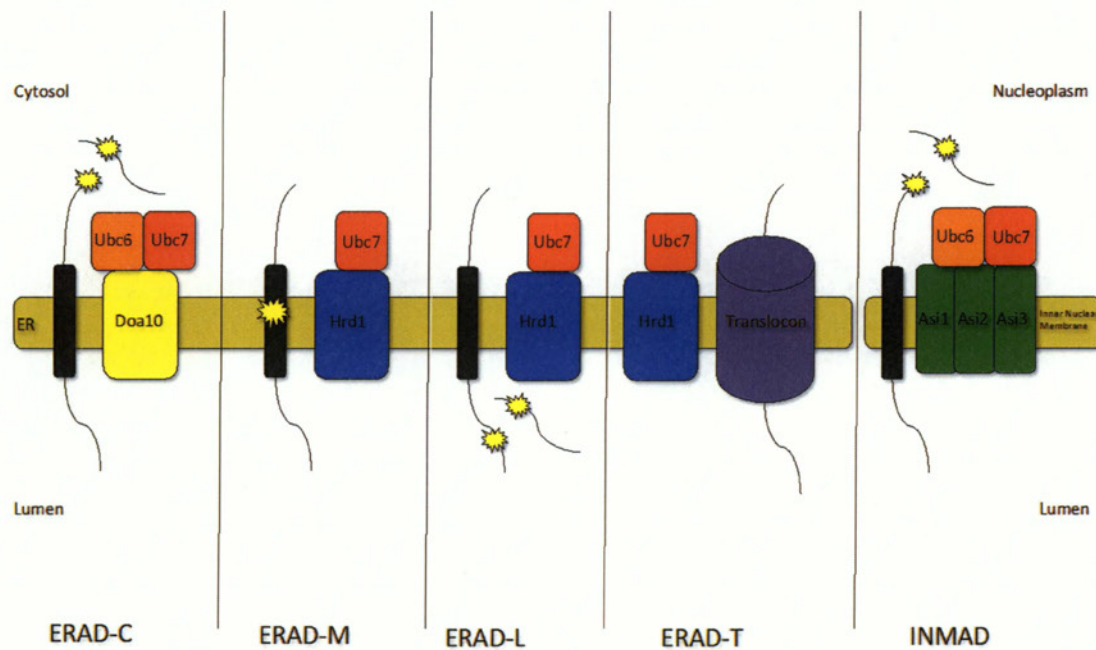


Figure 1. Degradation of proteins at the ER and inner nuclear membrane. The E3 ubiquitin ligases Doa10, Hrd1, and Asi complex target proteins for degradation by the proteasome. Doa10 catalyzes degradation of proteins with cytosolic degradation signals (ERAD-C substrates). Hrd1 catalyzes degradation of proteins with intramembrane and luminal degradation signals (ERAD-M and ERAD-L substrates) and proteins that aberrantly or persistently engage the translocon (ERAD-T substrates). The Asi complex (Asi1/Asi2/Asi3) catalyzes the degradation of inner nuclear membrane proteins. Yellow stars represent degradation signals.

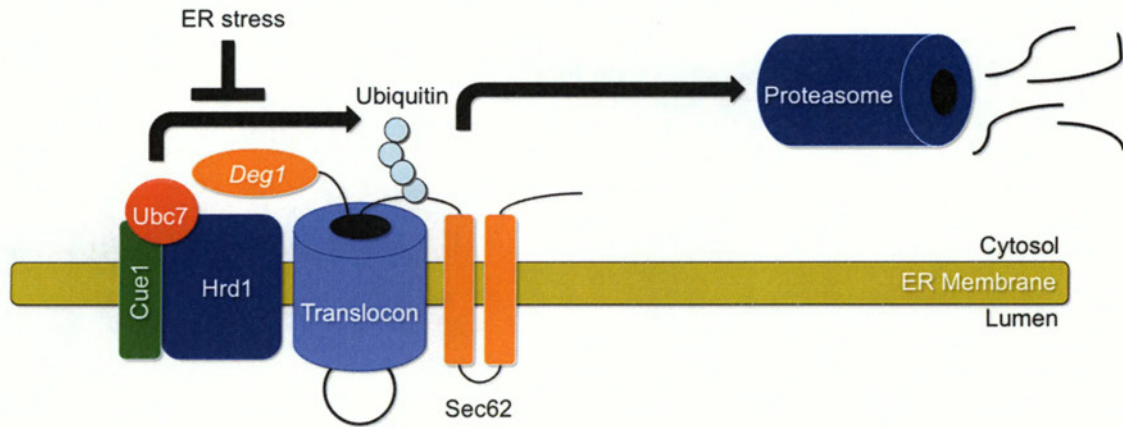


Figure 2. After aberrantly engaging the translocon, *Deg1*-*Sec62* becomes polyubiquitylated by Hrd1, a ubiquitin ligase, and Ubc7, a ubiquitin-conjugating enzyme. The protein is then sent to the proteasome for degradation. ER stress is an inhibitor of Hrd1-dependent degradation of *Deg1*-*Sec62*.

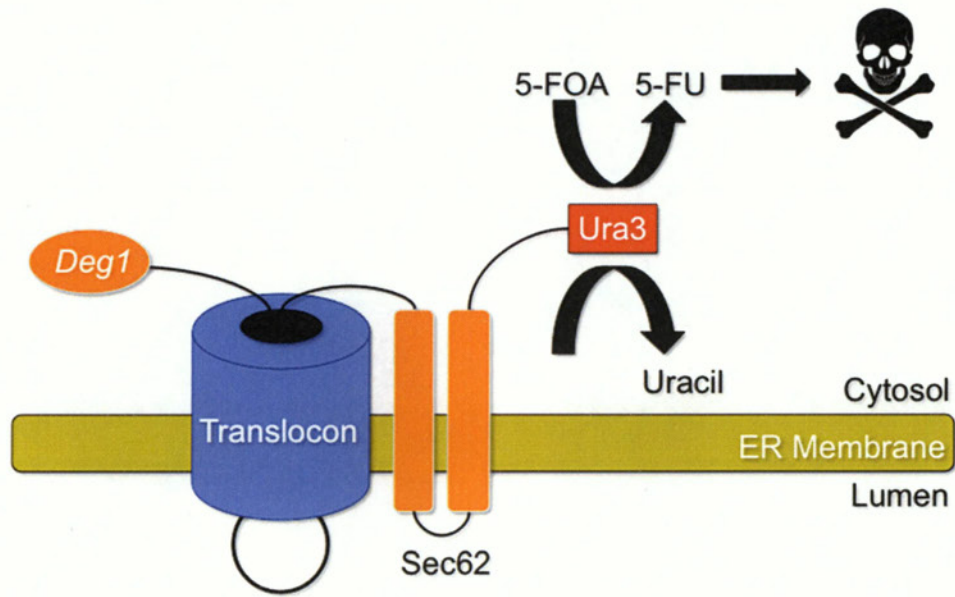


Figure 3. Fusion of the Ura3 enzyme to the C-terminus of *Deg1-Sec62* allows for protein degradation to be monitored on the basis of cellular growth. Cells in which *Deg1-Sec62-Ura3* protein is stabilized (e.g. cells lacking Hrd1 or experiencing ER stress) are able to synthesize uracil or convert 5-FOA to the toxic compound 5-FU. Cells grow in the absence of uracil if the degradation pathway is impaired and survive in the presence of 5-FOA if the degradation pathway is intact.

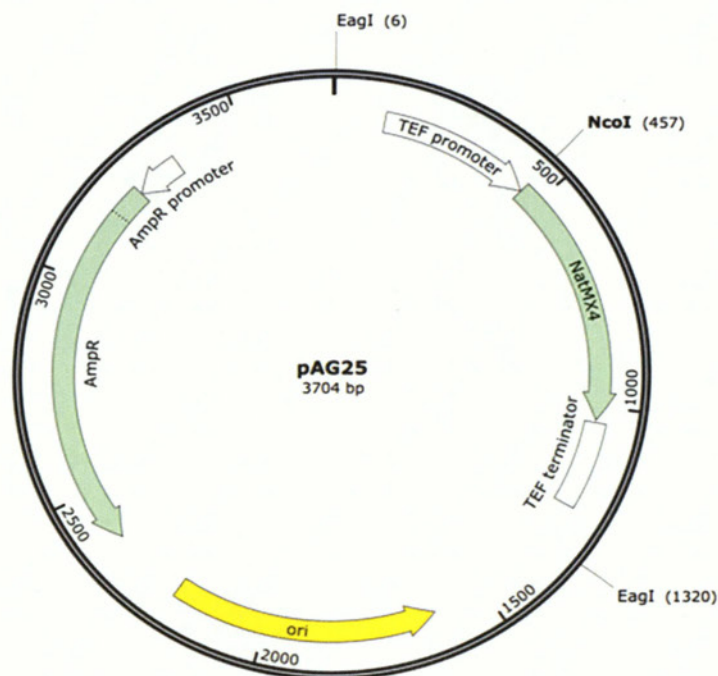


Figure 4. The plasmid pAG25 possesses the *natMX4* gene. Restriction sites for the enzymes *NcoI* and *EagI* are indicated. Digestion of this plasmid with *EagI* yielded the insert fragment containing *natMX4* for the generation of a plasmid containing both *Deg1*-*Sec62*-*Ura3* and *natMX4*. Amplification of the *natMX4* gene by PCR provided the DNA used to replace the *ASI3* gene in strain construction experiments.

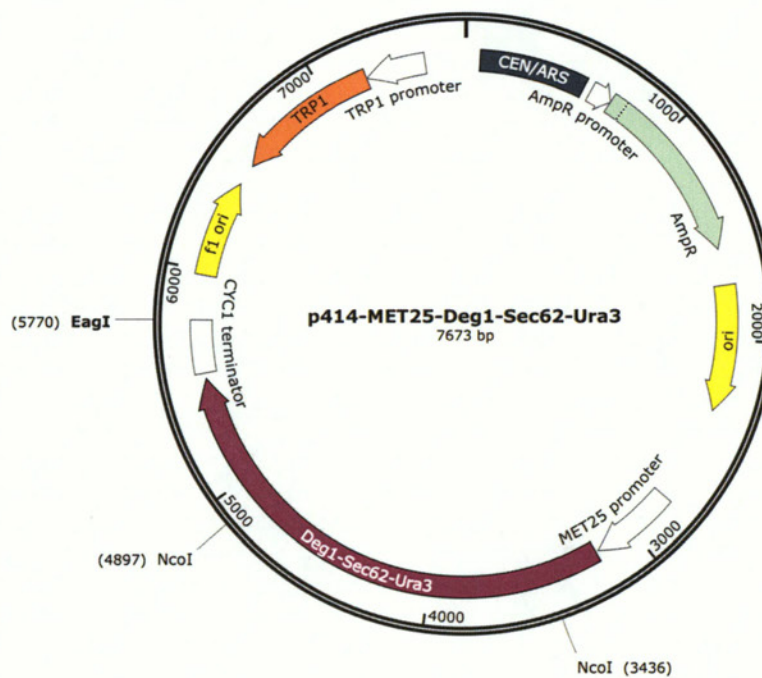


Figure 5. Following digestion with *EagI*, the plasmid p414-MET25-*Deg1*-Sec62-Ura3 served as the vector fragment for the generation of a plasmid containing both *Deg1*-Sec62-Ura3 and natMX4. Restriction sites for the enzymes *NcoI* and *EagI* are indicated.

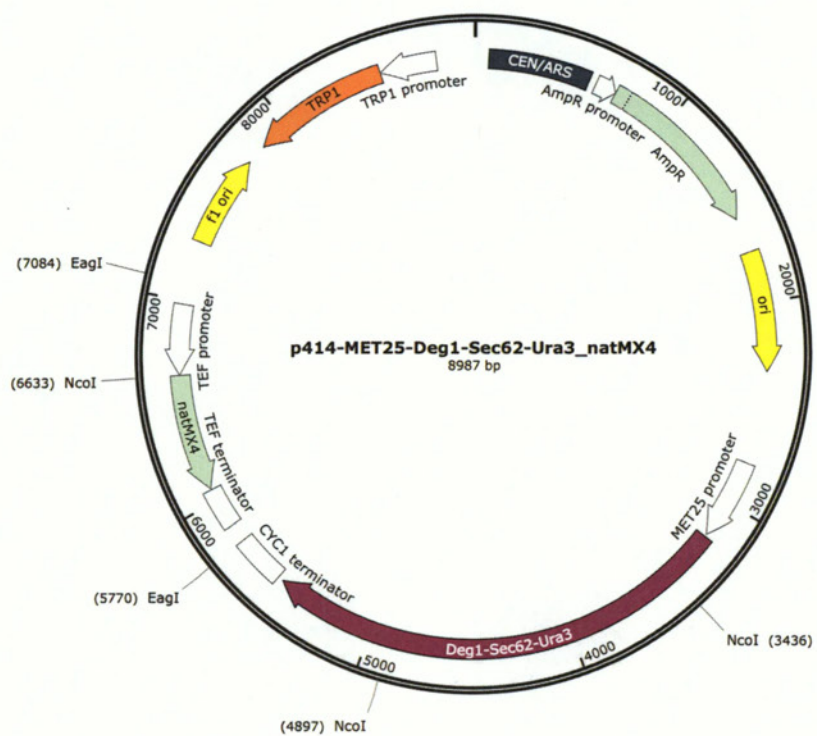


Figure 6. Recombinant plasmid molecule possessing vector (p414-MET25-*Deg1*-Sec62-Ura3) and insert (natMX4) fragments. Restriction sites for the enzymes NcoI and EagI are indicated.

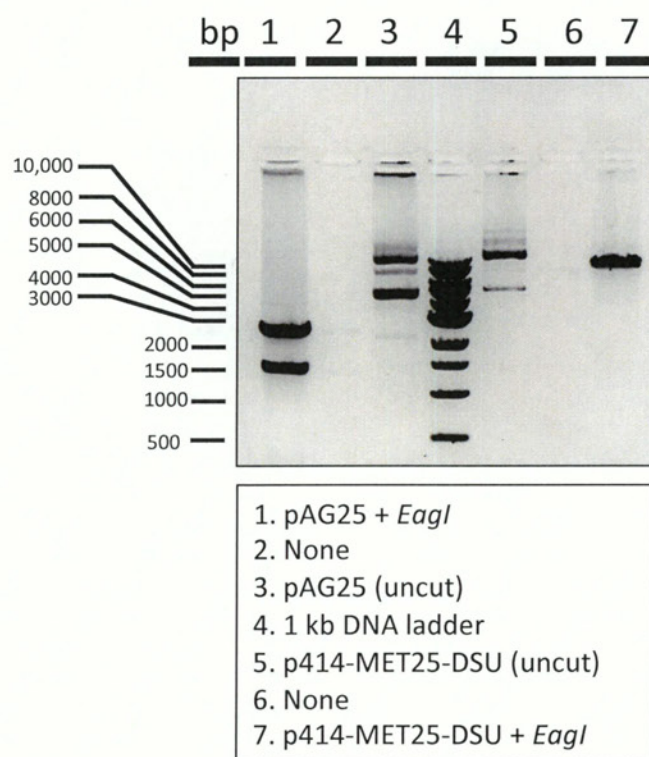


Figure 7. Agarose gel electrophoresis of the insert (pAG25) and vector (p414-MET25-*DegI*-Sec62-Ura3) fragments prepared by *EagI* digestion (and phosphatase treatment of the vector) for generation of recombinant plasmid molecule. Undigested vector and insert plasmids were included as controls. Expected fragment sizes: pAG25, 2390 and 1314 bp; p414-MET25-*DegI*-Sec62-Ura3, 7673 bp. The vector (7639 bp; Lane 7) and insert (1314 bp; Lane 1) fragments underwent gel purification and ligation.

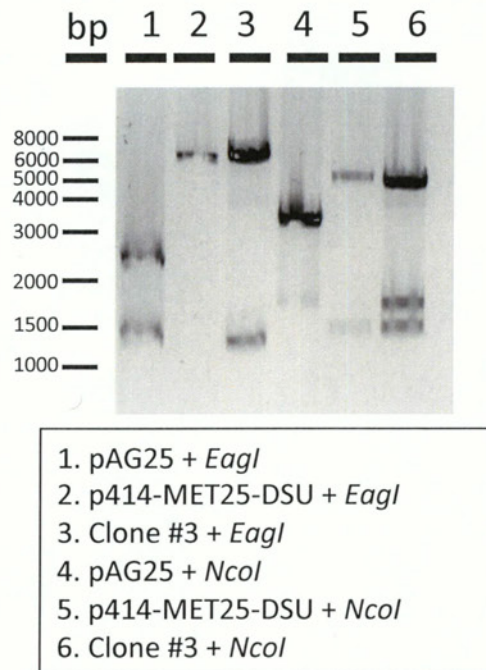


Figure 8. Agarose gel of *EagI* and *NcoI* restriction digests to screen candidate recombinant p414-MET25-*DegI*-Sec62-Ura3_natMX4 plasmid molecules. Expected *EagI* fragment sizes: p414-MET25-*DegI*-Sec62-Ura3 (parental vector plasmid), 7673 bp; pAG25 (parental insert plasmid), 2390 and 1314 bp; p414-MET25-*DegI*-Sec62-Ura3_natMX4 (recombinant plasmid), 7673 and 1314 bp. Expected *NcoI* fragment sizes: p414-MET25-*DegI*-Sec62-Ura3 (parental vector plasmid), 6212 and 1461 bp; pAG25 (parental insert plasmid), 3704 bp; p414-MET25-*DegI*-Sec62-Ura3_natMX4 (recombinant plasmid), 5790, 1736, and 1461 bp. Clone 3 is one successfully generated recombinant p414-MET25-*DegI*-Sec62-Ura3_natMX4 plasmid.



Figure 9. Agarose gel electrophoresis of natMX4 PCR-amplified from pAG25 and gel-purified. Amplified natMX4 possesses flanking sequences homologous to sequences upstream and downstream of the *ASI3* gene. The expected product size was 1464 bp.

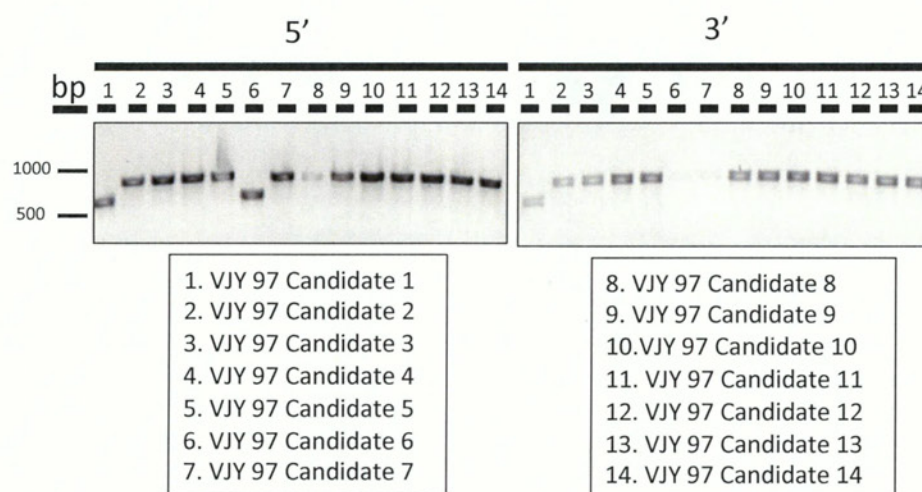


Figure 10. Assessment of *natMX4* integration into the *ASI3* locus of VJY97 via three-primer PCR. Left Panel: Three-primer PCR of the 5' end of insertion site. Expected sizes: *asi3Δ::natMX4*, 820 bp; WT *ASI3*, 649 bp. Right Panel: Three-primer PCR of the 3' end of insertion site. Expected sizes: *asi3Δ::natMX4*, 808 bp; WT *ASI3*, 623 bp.

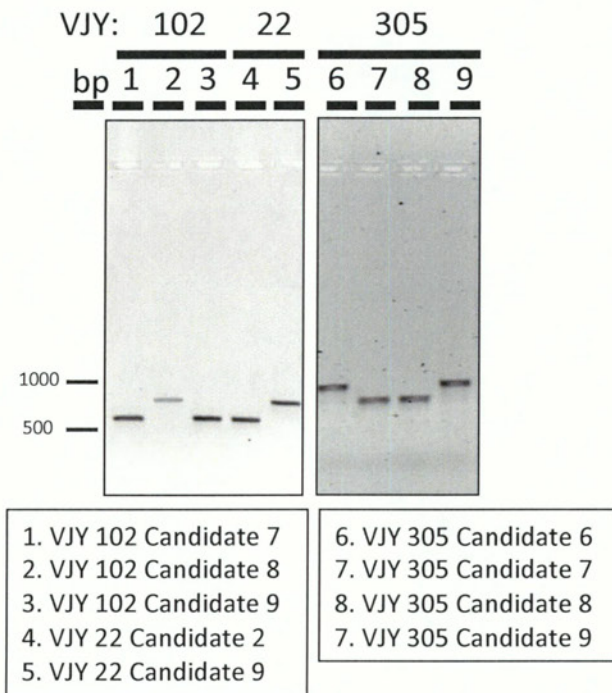


Figure 11. Assessment of natMX4 integration into the *ASI3* locus of VJY102, VJY22, and VJY305 via three-primer PCR at the 5' end of the insertion site. Expected sizes: *asi3Δ::natMX4*, 820 bp; WT *ASI3*, 649 bp.

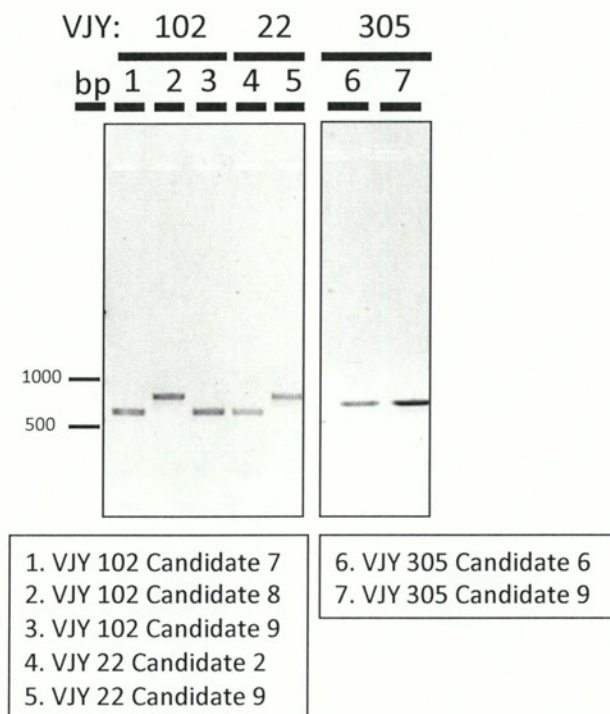


Figure 12. Assessment of natMX4 integration into the *ASI3* locus of VJY 102, VJY22, and VJY305 via three-primer PCR at the 3' end of the insertion site. Expected sizes: *asi3Δ::natMX4*, 808 bp; WT *ASI3*, 623 bp.

Table 1. Yeast strains used in this study.

Yeast Strains	Description	Genotype
VJY 22	<i>hrd1Δ</i> (<i>HRD1</i> knockout)	
VJY 97	Laboratory wild-type	
VJY 102	<i>doa10Δ</i> (<i>DOA10</i> knockout)	
VJY 305	<i>hrd1Δ doa10Δ</i> (<i>HRD1</i> and <i>DOA10</i> knockout)	
VJY 397	<i>asi3Δ</i> (<i>ASI3</i> knockout) – 97 candidate # 2	
VJY 398	<i>asi3Δ</i> (<i>ASI3</i> knockout) – 97 candidate # 3	
VJY 409	<i>hrd1Δ asi3Δ</i> (<i>HRD1</i> and <i>ASI3</i> knockout) – 22 candidate # 9	
VJY 410	<i>doa10Δ asi3Δ</i> (<i>DOA10</i> knockout) – 102 candidate # 8	
VJY 411	<i>hrd1Δ doa10Δ asi3Δ</i> (<i>HRD1</i> , <i>DOA10</i> , and <i>ASI3</i> knockout) – 305 candidate #6	
VJY 412	<i>hrd1Δ doa10Δ asi3Δ</i> (<i>HRD1</i> , <i>DOA10</i> , and <i>ASI3</i> knockout) – 305 candidate #9	

Table 2. Sequences of the PCR primers used in this study.

PCR Primer Numbers	Sequence (5'→3')
VJR246	ACAATTCAACGCGTCTGTGA
VJR249	ACCTCTGGCTGGAGGTCAC
VJR274	AGGAACAGTCATTACGTAGGGATTTCAAAGTTTGACTGCACATACGATTTAGGTGACAC
VJR275	TCCTATGATGTCTTAAATACGTATACCTAATAAAATAATTAATACGACTCACTATAGGGAG
VJR276	TCCAAACAATTGATGGCAGA
VJR277	TCAATGCTTGAAAAGAGTCCAA
VJR278	TGAACGCCTTCTGCATCATA
VJR279	TTTGGAACTTACTAGAAAAGGCTA

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